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TECHNICAL NOTES

Hepatoma G2 conditioned medium facilitates early outgrowth of endothelial cells from isolated glomeruli

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Since it was first shown that glomerular cells could be propagated in culture [1], this technique has proven to be an invaluable tool for studying the physiology of individual glomerular cells. Epithelial, mesangial, and endothelial cells have been isolated and propagated in several species [2], however, the population of cells obtained consisted of more than one of these glomerular cell types. The limited availability of normal human kidney specimens for tissue culture has led to attempts to optimize culture conditions which could selectively enhance the growth of one specific cell type. Hoshi and McKeehan [3] have demonstrated that medium conditioned by HepG2 cells, a hepatoblastoma line, contained one or more soluble growth factors which favored the growth of human umbilical vein endothelial cells in serum free media.

This laboratory has previously reported the growth of human glomerular endothelial cells in the presence of fetal bovine serum supplemented with platelet-derived growth factor [4]. In view of our continued interest in these cells, and the variation in the ability of different lots of fetal bovine serum to support endothelial cell growth, we investigated the use of hepatoma conditioned medium to further optimize the growth of endothelial cells.

Methods

Isolation of glomeruli

The glomeruli were obtained from human kidneys removed for renal cancer. Cortical tissue, far from the tumor zone were stripped of capsule, and the fragments diced into approximately 2 mm^3. The pieces were gently forced through a single stainless steel mesh (80, E.C. Apparatus, St. Petersburg, Florida, USA) with a glass pestle. Only material passing easily through this single mesh size was collected in Hanks media at 37°C (Quality Biological, Inc., Gaithersburg, Maryland, USA) and allowed to settle in 50 ml conical tubes (Falcon). Generally, recognizable glomeruli represented more than 80% of the structures in the initial preparation of sieved material. As intact glomeruli appeared to settle faster than non-glomerular contaminants, the preparations were resuspended several times in fresh medium and allowed to settle a minimum of five times. The supernatant was aspirated each time, effectively removing non-glomerular contaminants.

This procedure was repeated until the preparation was free of contaminants. This relatively gentle method of isolating glomeruli was found to require a short period of time and resulted in isolates with better plating efficiency. Encapsulated glomeruli were not observed visually.

Cell culture

Primary cultures. Glomeruli were plated in 100 mm dishes (Nunclon) coated with human fibronectin (200 μg/ml, Collaborative Research, Lexington, Massachusetts, USA). The glomerular pellet was divided and plated in either Waymouth's medium (Gibco, Grand Island, New York, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco), and 2 ng/ml of platelet-derived growth factor (PDGF) (BRL, Gaithersburg, Maryland, USA) (Medium A), a 1:1 mixture of hepatoma G2 conditioned medium (CM) and Waymouth's medium with 20% FBS (Medium B), or unconditioned MDCB medium with 15% FBS (Medium C). All media contained penicillin (100 U/ml), streptomycin (100 μg/ml, Gibco) and glutamine (1 mM, Gibco). The dishes were placed in an incubator at 37°C and cultured in a 5% CO_2 and 95% air mixture.

Hepatoma conditioned medium (CM)

Confluent human hepatoblastoma cells, HepG2 (ATCC #HB8065, Rockville, Maryland, USA) were maintained in T175 flasks (Nunclon), in medium MDCB 107 supplemented with 10% fetal bovine serum as described previously [3]. Conditioned medium was collected every three days from confluent monolayers, centrifuged at 3000 rpm for 15 minutes to remove cell debris, and frozen at −80°C for periods not exceeding one month.

Outgrowth. Dishes were examined at day 7 after the initial plating, and glomeruli which had not attached and non-glomerular fragments were aspirated. At day 7 fresh medium was added to the dish irrespective of the cell number. Once a week thereafter the medium was replaced. At confluence, the cells were passaged and counted.

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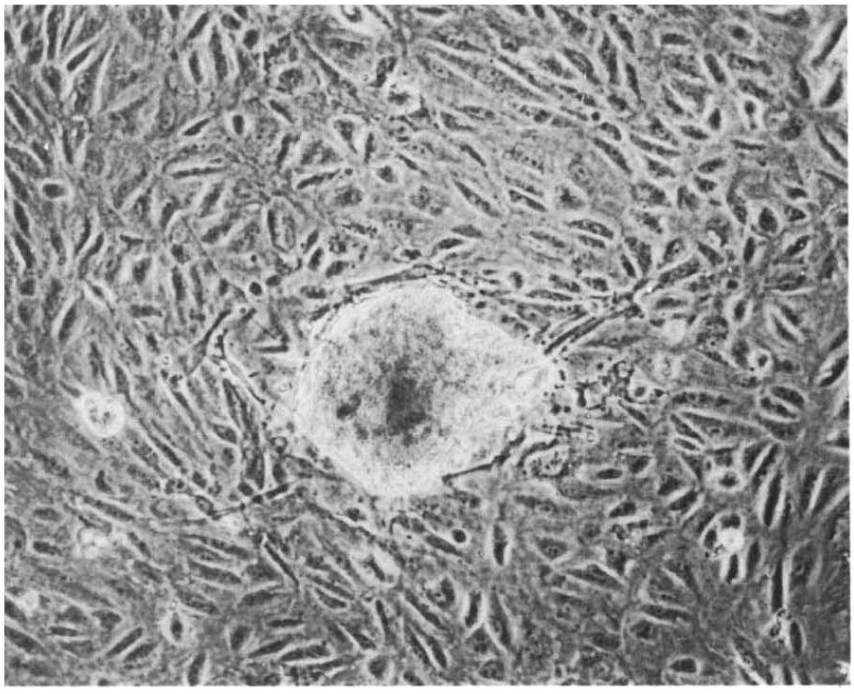

Fig. 1. Primary outgrowth in medium A (×100).

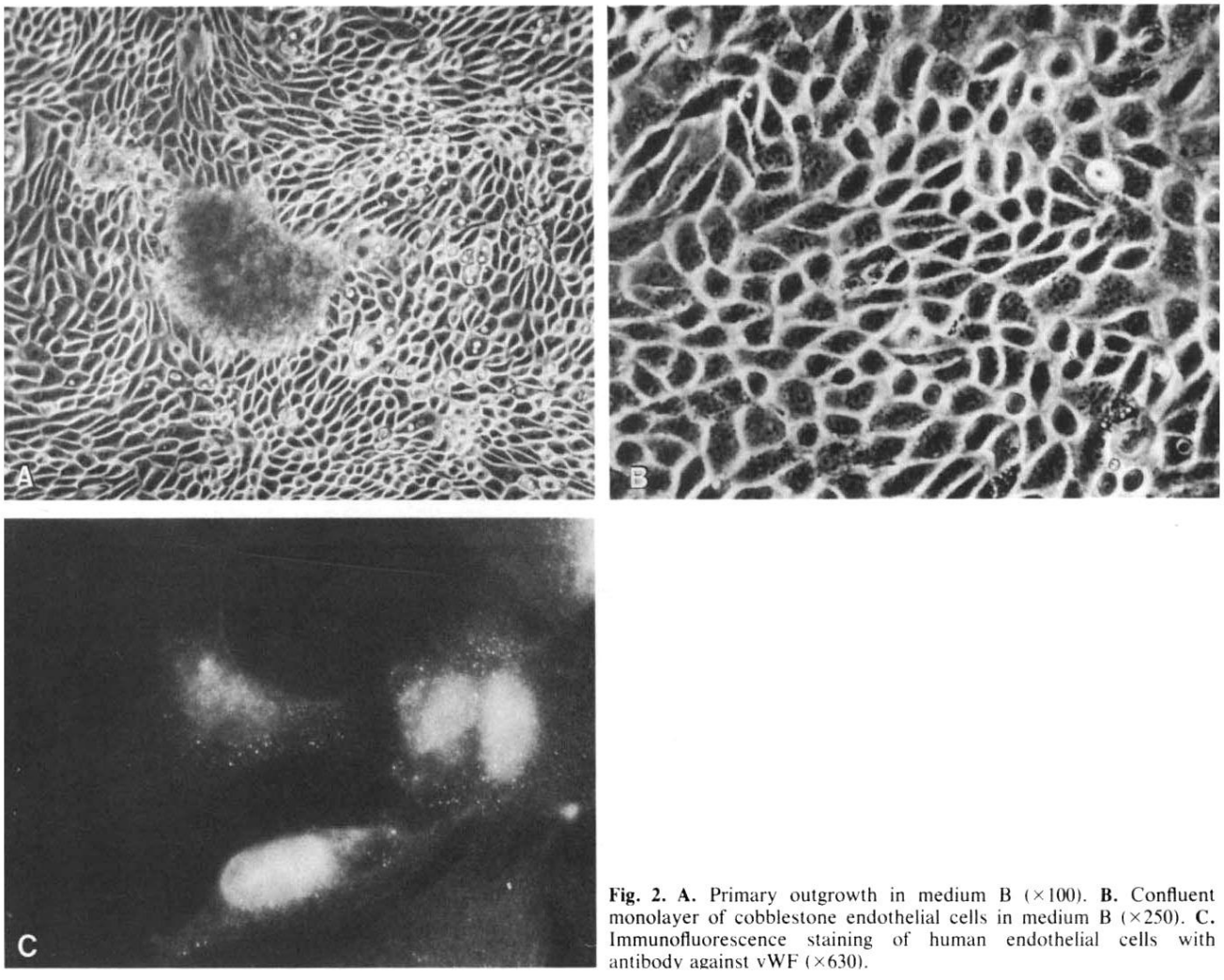

Fig. 2. A. Primary outgrowth in medium B (×100). **B.** Confluent monolayer of cobblestone endothelial cells in medium B (×250). **C.** Immunofluorescence staining of human endothelial cells with antibody against vWF (×630).

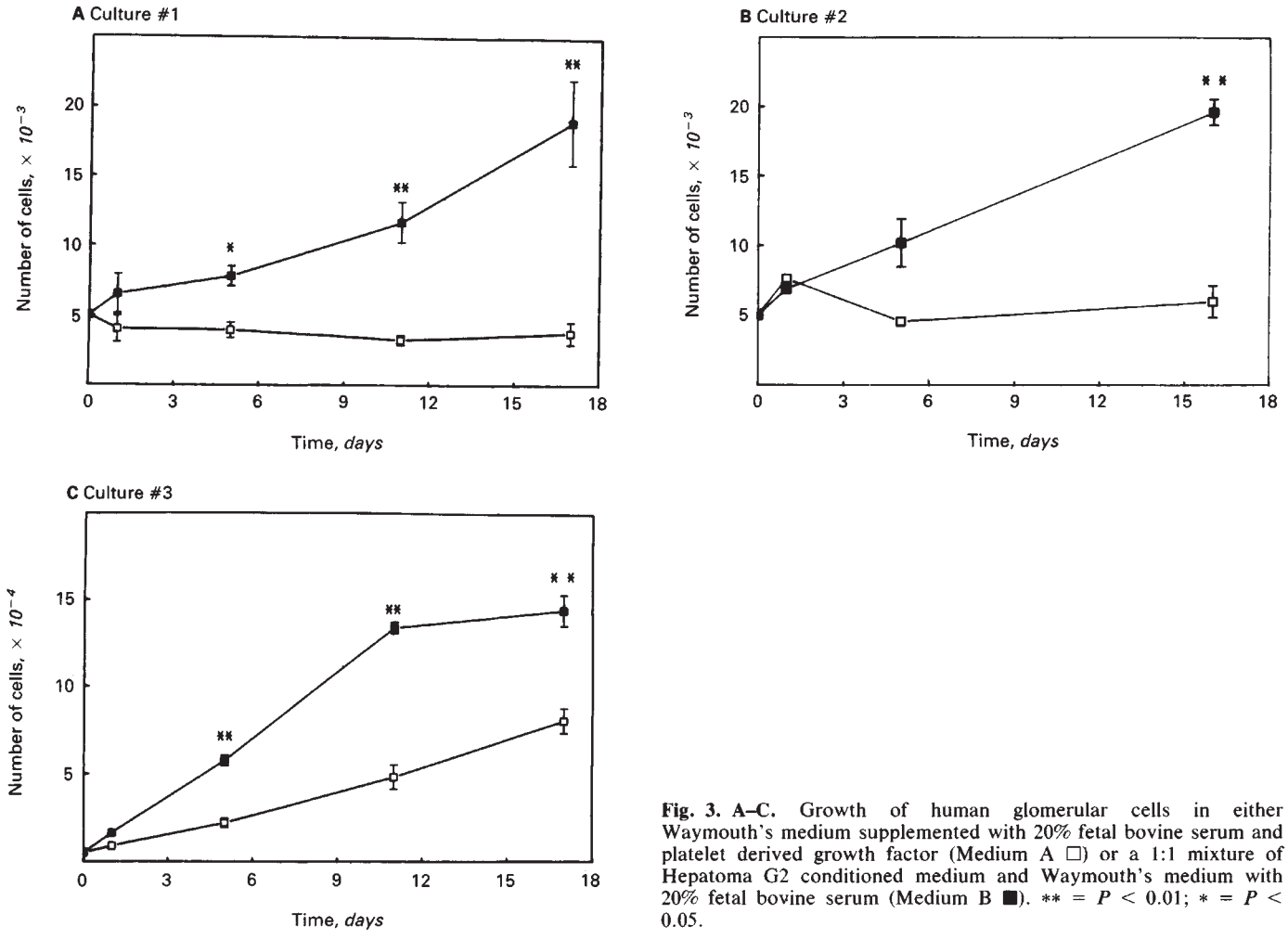

Fig. 3. A–C. Growth of human glomerular cells in either Waymouth's medium supplemented with 20% fetal bovine serum and platelet derived growth factor (Medium A □) or a 1:1 mixture of Hepatoma G2 conditioned medium and Waymouth's medium with 20% fetal bovine serum (Medium B ■). ** = $P < 0.01$; * = $P < 0.05$.

Growth curves

Growth curves were performed on cells at passage 2 in both A and B media. Equal numbers of cells were plated in 24 well plates (Nunclon) and then trypsinized and counted in triplicate (Particle Data Counter) on day 1, 5, 11, and 17. The media were changed on day 7.

Fluorescence microscopy

At the time of plating for growth curves, cells were plated in their respective media on fibronectin-coated Meloy glass slides (Meloy Labs) and eight-well plastic chamber slides (Lab-Tek) at a density of 10,000 cells or 2000 cells/well, respectively. Cells plated on Meloy slides were stained on day 1 of the growth curves, while those on chamber slides were used for staining on day 17, and were fed on day 7. Briefly, the slides were rinsed in PBS, fixed in 2% paraformaldehyde at 37°C for 10 minutes and rinsed in PBS. Fixed cells were permeabilized with 0.1% triton X-100 and incubated with normal goat serum for 15 minutes prior to staining. The cells were exposed to the first antibody for 30 minutes at room temperature, and then washed in PBS. The second antibody, raised in goat and FITC-conjugated, was applied to the cells with an incubation of 30 minutes in the dark. The cells were rinsed in PBS and mounted using a mixture of p-phenylendiamine (1 mg/ml) dissolved in PBS glycerol. The antibodies used were directed against von Willebrand factor VIII-related antigen (vWF) (Calbiochem-Behring Corps., La Jolla, California, USA) a marker of endothelial cells, cytokeratin (Enzo-Biochem Inc., New York, New York, USA), a marker of epithelial cells, FITC-conjugated anti-rabbit IgG (Tago, Burlingame California, USA), FITC-conjugated anti-mouse IgG (Tago), and the toxin phalloidin rhodamine-labeled (Molecular Probes, Inc., Eugene, Oregon, USA) which binds to actin microfilaments. The IgG fraction of the antibody against vWF was purified with DEAE Affi-Gel Blue Chromatography (BioRad, Richmond, California, USA).

Acetylated LDL

Uptake of acetylated low-density lipoprotein (LDL) [5] was used as an additional criteria to assess the percentage of endothelial cells. Cells were plated at confluence on a 4-well chamber slide and incubated with 10 μg/ml 1,1 1-dioctadecyl-1-3,3,3′,3′- tetramethyl-indocarbocyanine perchlorate labeled acetylated LDL (Biomedical Technologie Inc., Stoughton, Massachusetts, USA) in respective medium for four hours at 37°C. The cells were washed, mounted, and positive cells counted.

Results

It has been previously shown that the cellular composition of the outgrowth from human glomeruli follows a predictable sequence using a single medium. Visceral epithelial cells, predominant during the first two weeks, are gradually overtaken and largely replaced by mesangial cells in primary culture. Endothelial cells could only be propagated in the presence of serum, supplemented with PDGF [4]. Under these conditions endothelial cells rather than epithelial cells were the prominent cell in the early outgrowth of unpassaged cells (Fig. 1). As each lot of fetal calf serum yields markedly different results in the support of fastidious cell populations, we sought a modification of growth conditions not only to select at primary culture for endothelial cells, but to support their continued growth with passage. HepG2 cells synthesize and release into the medium an array of growth factors which stimulate the replication of glomerular as well as human umbilical vein cells [3]. We found that medium conditioned by HepG2 cells enriched the glomerular cell outgrowth for endothelial cells (Figs. 2A, B). Three different human kidneys were examined (Figs. 3A, B, C). The increase in growth obtained with medium B was statistically significant for all specimens on day 17. (Kidneys 1 and 2 showed a 3.6-fold increase in growth in medium B as opposed to medium A. Kidney 3 showed a 1.8-fold increase in medium B.) Unconditioned MDCB supported no growth and provided poor attachment of glomeruli. Numerous vWF positive cells, that is, endothelial cells, were identified in all cultures and both media. However, those grown in medium B contained between 85% to 95% vWF positive cells (Fig. 2C), while those grown in medium A contained 50% to 85% positive cells. Approximately 10% of cells contained cytokeratin, a pattern consistent with epithelial cells [2]. Further evidence was obtained by the large proportion of cells in medium B which were positive for uptake of acetylated LDL, whereas there were fewer cells positive for acetylated LDL in medium A. The initial outgrowth of the glomeruli plated in medium B appeared more abundant, and the number of cells at passage one in medium B was twice that of medium A for Kidney 1 and 3. As has been reported, HepG2 conditioned media contains ECGF 2a and 2b, which are both proteinase inhibitors [6]. Whether these or other growth factors are responsible for the observed endothelial cell responsiveness has yet to be elucidated. In conclusion, we found the use of this medium supports and maintains human glomerular endothelial cells in culture.

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